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 JC644 U.S. PTO 09/10/99	<b>UTILITY PATENT APPLICATION TRANSMITTAL</b> <b>(Large Entity)</b> <i>(Only for new nonprovisional applications under 37 CFR 1.53(b))</i>	Docket No. P-4762
		Total Pages in this Submission 42

**TO THE ASSISTANT COMMISSIONER FOR PATENTS**

Box Patent Application  
 Washington, D.C. 20231

Transmitted herewith for filing under 35 U.S.C. 111(a) and 37 C.F.R. 1.53(b) is a new utility patent application for an invention entitled:

**A COMPOSITION FOR PROVIDING LONG TERM STABILITY TO CELLS FOR DIAGNOSTIC TESTING**

and invented by:

**DOLORES M. BERGER, DARETTA A. YURISIS, WILLIAM A. NUSSBAUMER and ANNE B. BROWN**

If a **CONTINUATION APPLICATION**, check appropriate box and supply the requisite information:

☒ **Continuation** ☐ **Divisional** ☐ **Continuation-in-part (CIP)** of prior application No.: \_\_\_\_\_

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Enclosed are:

**Application Elements**

1. ☒ Filing fee as calculated and transmitted as described below
2. ☒ Specification having 27 pages and including the following:
  - a. ☒ Descriptive Title of the Invention
  - b. ☐ Cross References to Related Applications *(if applicable)*
  - c. ☐ Statement Regarding Federally-sponsored Research/Development *(if applicable)*
  - d. ☐ Reference to Microfiche Appendix *(if applicable)*
  - e. ☒ Background of the Invention
  - f. ☒ Brief Summary of the Invention
  - g. ☒ Brief Description of the Drawings *(if drawings filed)*
  - h. ☒ Detailed Description
  - i. ☒ Claim(s) as Classified Below
  - j. ☒ Abstract of the Disclosure

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Total Pages in this Submission  
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## Application Elements (Continued)

3. ☒ Drawing(s) (when necessary as prescribed by 35 USC 113)
- a. ☐ Formal                      Number of Sheets \_\_\_\_\_
- b. ☒ Informal                      Number of Sheets 6
4. ☒ Oath or Declaration
- a. ☐ Newly executed (original or copy)                      ☒ Unexecuted
- b. ☐ Copy from a prior application (37 CFR 1.63(d)) (for continuation/divisional application only)
- c. ☒ With Power of Attorney                      ☐ Without Power of Attorney
- d. ☐ DELETION OF INVENTOR(S)  
Signed statement attached deleting inventor(s) named in the prior application,  
see 37 C.F.R. 1.63(d)(2) and 1.33(b).
5. ☐ Incorporation By Reference (usable if Box 4b is checked)  
The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.
6. ☐ Computer Program in Microfiche (Appendix)
7. ☐ Nucleotide and/or Amino Acid Sequence Submission (if applicable, all must be included)
- a. ☐ Paper Copy
- b. ☐ Computer Readable Copy (identical to computer copy)
- c. ☐ Statement Verifying Identical Paper and Computer Readable Copy

## Accompanying Application Parts

8. ☐ Assignment Papers (cover sheet & document(s))
9. ☐ 37 CFR 3.73(B) Statement (when there is an assignee)
10. ☐ English Translation Document (if applicable)
11. ☐ Information Disclosure Statement/PTO-1449                      ☐ Copies of IDS Citations
12. ☐ Preliminary Amendment
13. ☒ Acknowledgment postcard
14. ☒ Certificate of Mailing
- ☐ First Class                      ☒ Express Mail (Specify Label No.): EL 416960593US

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## Accompanying Application Parts (Continued)

15. ☐ Certified Copy of Priority Document(s) (if foreign priority is claimed)

16. ☐ Additional Enclosures (please identify below):

## Fee Calculation and Transmittal

### CLAIMS AS FILED

For	#Filed	#Allowed	#Extra	Rate	Fee
Total Claims	17	- 20 =	0	x \$18.00	\$0.00
Indep. Claims	1	- 3 =	0	x \$78.00	\$0.00
Multiple Dependent Claims (check if applicable) <input type="checkbox"/>					\$0.00
BASIC FEE					\$760.00
OTHER FEE (specify purpose)					\$0.00
TOTAL FILING FEE					\$760.00

- ☐ A check in the amount of \_\_\_\_\_ to cover the filing fee is enclosed.
- ☒ The Commissioner is hereby authorized to charge and credit Deposit Account No. 02-1666 as described below. A duplicate copy of this sheet is enclosed.
- ☒ Charge the amount of \$760.00 as filing fee.
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- ☒ Charge any additional filing fees required under 37 C.F.R. 1.16 and 1.17.
- ☐ Charge the issue fee set in 37 C.F.R. 1.18 at the mailing of the Notice of Allowance pursuant to 37 C.F.R. 1.311(b).

  
Signature

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## A Composition for Providing Long Term Stability to Cells for Diagnostic Testing

By: Dolores M. Berger, Daretta A. Yursis, William A. Nussbaumer and Anne B. Brown

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### FIELD OF THE INVENTION

10 The present invention relates to a method and composition for stabilizing cells in a sample (such as, for example, a clinical specimen in a biological sample) for transport and subsequent testing for diagnosis. The composition for stabilizing the cells is specifically capable of maintaining nucleic acids intact for hybridization with oligonucleotide capture and detector probes.

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### BACKGROUND OF THE INVENTION

20 Diagnostic testing of infectious and sexually transmitted diseases has become increasingly focused on faster, more accurate results. Nucleic acid probe technology has enabled rapid diagnostic testing to break time-to-result barriers with high specificity, and less subjectivity. While they are faster than growth based, biochemical assays, and more specific than immunologically based assays, nucleic acid probe assays present a unique challenge for delivering the target sample intact. Furthermore, samples that may be collected at one site, and tested at another site, are particularly vulnerable to nucleic acid degradation if not handled properly.

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30 Nucleic acid detection by hybridization and capture has been applied to a host of diseases. It has been especially useful for infectious diseases in which conventional methods are time consuming, expediency of treatment is critical, and/or the disease is reportable to health agencies. *Trichomonas vaginalis* vaginitis (trichomoniasis) is a reportable sexually transmitted disease that affects approximately 3 million women per year in the U.S. Furthermore, vaginal disorders due to bacterial vaginosis (BV) and candidiasis, are two of the most common reasons women seek medical treatment. The symptoms of these three distinct diseases overlap, thus creating a need for differential diagnosis before appropriate and specific medication can be prescribed. A rapid and accurate diagnosis is especially critical in pregnant women, in whom BV and trichomoniasis are associated with premature births and low birth weight babies. Moreover, BV-positive pregnant women are predisposed to chorioamnionitis, amniotic fluid infection, and puerperal infectious morbidity. BV has also been associated with pelvic inflammatory disease, postpartum endometritis, bacteremia, salpingitis, and the like. Proper diagnosis and treatment of vaginitis requires identifying the causative microorganism so that the appropriate antimicrobial treatment can be defined.

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The Affirm VPIII nucleic acid hybridization assay, described in U.S. Patent No. 5,654,418, is a significant advance in the diagnosis of vaginitis, due to its ability to detect *T. vaginalis*, *G. vaginalis* or *C. albicans*, from a single vaginal swab. The swab is incubated in a lysis solution at high temperature, which causes the organism to lyse and release nucleic acid. A buffer solution is then added to the sample. The sample solution is next incubated with a set of nylon beads that are each derivatized with specific capture probes. The rRNA hybridizes to the capture beads, which are next incubated with a solution containing biotinylated detector oligonucleotide probes. The detector probes hybridize to another region in the rRNA. The bead is transferred to a well containing an enzyme. If biotinylated detector probes are hybridized to the rRNA, the enzyme will bind to the biotin. If there is no rRNA hybridized to the bead, no biotinylated detector probe will be present for the enzyme to bind. Finally, the beads are incubated with a substrate, which will react with the enzyme to form a blue color. If rRNA is present the beads will appear blue. If there is no rRNA in the sample, the beads remain colorless. A differential diagnosis can be obtained from a single sample by using three beads, each bead specific for only one of the analytes.

The present invention was developed to provide stability to vaginal swab samples, specifically, samples collected to test for the presence of *Candida*, *Gardnerella* or *Trichomonas*, using the Affirm VPIII Microbial Identification Test. Without the aid of the present invention, the swab samples will only remain stable for up to one hour at ambient temperature, or four hours at refrigerated temperature. Specifically, the rRNA within the cells must remain intact in order to be detected, and, the presence of low, non-pathological numbers of *Candida* must be kept from multiplying and producing a false positive. The proper preservative would allow for sample collection and sample testing to be conducted at remote sites, or for numbers of samples to be batched for processing and testing all at once.

The appropriate transport or preservative or fixative solution for the Affirm VPIII sample needed to have the following attributes:

1. The solution had to control or inhibit RNA degrading enzymes (RNases) found in vaginal fluid.
2. It had to prevent growth of *Candida*, *Gardnerella* or *Trichomonas*, while...
3. controlling RNA degradation within the cells due to endogenous nucleases or cell death.
4. The solution had to be compatible with the Affirm VPIII test *as embodied*.
5. It could not introduce unnecessary risk to the end users, and
6. It would provide signal stability for samples stored up to 72 hours.

Conventional preservatives, such as those having bactericidal or inhibitory effects, prevent growth of low levels of organisms, but do not address the problem of nucleic acid degradation. Conversely, transport media tend to be minimal or starvation media, formulated to maintain viability of the organism for culture later. *Candida*, however, tend to flourish in such media, while *Gardnerella* and *Trichomonas* do not survive. The

result with such media is a false positive Affirm result for *Candida* and a false negative for the latter two. The complexity of the problem is increased by the fact that these three organisms represent both prokaryotic and eukaryotic cell types, and each has a distinctly constructed cell wall and/or membrane.

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Fixatives, as a class of substances, tend to contain alcohol, formaldehyde or chloroform, and a wide range of additives, depending on the specimen and application. Many are not stable solutions, suitable only for use within hours of preparation. Furthermore, they may present hazards beyond those already faced by the clinician (i.e.,  
10 mercuric chloride, picric acid). Formaldehydes were found to be incompatible with Affirm reagents. Alcohol based fixing agents offered the most promise due to their ability to precipitate or denature proteins, particularly, nucleases.

One such fixative is described in U.S. Patent No. 5,256,571, for preserving the  
15 structure of mammalian cells. Hurley et al claim a solution (designated from here on as PreservCyt) of 45 to 55% methanol, an anti-clumping agent and a buffering agent. However effective this solution may be for mammalian cells, it was not capable of meeting the criteria outlined above for all three vaginal pathogens of interest. This was most likely due to the added complexity of the cell wall structure of each organism, a  
20 structure not encountered in mammalian cells. Furthermore, in these studies, increasing the methanol concentration to 95% did not preserve, or fix, the vaginal samples such that the rRNA was detectable after 24 hours.

Many fixatives used for cytology and histology are home-brewed solutions, the  
25 formulations of which are well known to those skilled in the art. They are frequently prepared fresh and used within a short period of time, as mentioned above. Such solutions may include for example: 10% neutral buffered formalin, Carnoy's solution (ethanol, chloroform, acetic acid), B-5 (mercuric chloride, sodium acetate, formalin, water), Bouin's solution (picric acid, glacial acetic acid, formaldehyde), and Zenker's  
30 solution (water, potassium dichromate, mercuric chloride, glacial acetic acid). These formulations are routinely published in reference pages, via the internet, by academic or research institutions such as: the University of Bristol's Department of Pathology & Microbiology (<http://lang-dl-srv.lang.bris.ac.uk/Cpl/histfix.htm>), The Jackson Laboratory (<http://www.jax.org/resources/documents/sss/imaging/histf.html>), and the University of  
35 Newcastle at Australia ([http://www.newcastle.edu.au/departement/bi/birjt/techinfo/bio\\_fix.html](http://www.newcastle.edu.au/departement/bi/birjt/techinfo/bio_fix.html)). One lesser known fixative, published via the internet by the University of Texas, Austin ([http://vize222.zo.utexas.edu/Marker\\_pages/methods\\_pages/fixatives.html](http://vize222.zo.utexas.edu/Marker_pages/methods_pages/fixatives.html)), is known as Dents solution. This solution contains four parts methanol and one part dimethyl sulfoxide (DMSO). Dents solution is described as a fixative used for  
40 immunological staining of *Xenopus* specimens. The specified protocol is to fix samples at -20°C overnight. The authors claim that samples prepared in this manner may be stable frozen for many months, perhaps years.

It has been found by the inventors that the above described Dents solution will preserve vaginal swab samples containing vaginal fluid and seeded quantities of *Trichomonas vaginalis*, *Gardnerella vaginalis*, and *Candida albicans*. Swabs stored in this solution are stable for several days at *ambient temperature*, prior to testing in the Affirm VPIII system. Results obtained with such swabs give similar signals to identical swabs tested immediately after preparation. It has also been found by the inventors that ethanol, or mixtures of ethanol and methanol, will also preserve the samples when mixed with DMSO. The preferred embodiment uses a 1:1 mix of methanol and DMSO.

Dimethyl sulfoxide has been used as an ingredient mixed with other molecules in previous references but in significantly smaller concentrations. U.S. Patent No. 5,622,867 uses 0.5 to 6% DMSO in a solution to store blood platelets; U.S. Patent No. 3,852,155 uses 8 to 10% DMSO in a solution for cryopreserving equine cell cultures; U.S. Patent No. 5,364,756 describes a solution containing 0.5M (~3-4%) DMSO. U.S. Patent Nos. 5,422,277 and 4,666,699 use DMSO in stain-fixative solutions at 5 to 10% and 3 to 8% respectively. However, in every one of these instances, DMSO is only a small fractional component of a much more complex solution.

In the present invention, the inventors describe a novel composition which is a mixture of a first substance which is at least one alcohol or ketone and a second facilitating substance such as DMSO, with a preferred embodiment being a mixture of 50% methanol/50% DMSO, and the method of providing long term (several days) stability, for example, to cells and in particular, clinical specimens, utilizing this composition.

Specifically, in a preferred embodiment, the clinical specimens are considered to be vaginal swabs, containing the causative agents for vaginitis and bacterial vaginosis, however, the solution could be used for other biological specimens in which the recovery of RNA is necessary. This solution will be useful in preventing the degradation of nucleic acids (i.e., DNA, RNA) located within cells, suspended in a matrix of biological fluid such as vaginal fluid. Furthermore, this solution will be capable of preventing degradation of, for example, RNA, an easily degraded nucleic acid, over several days at ambient temperatures and above.

## SUMMARY OF THE INVENTION

The present invention provides a novel cellular fixative composition and method of cell (and further, clinical specimen) preservation.

In a preferred embodiment, the composition of the present invention is comprised of a first substance capable of precipitating or denaturing proteins; and a second facilitator substance to aid in the infusion of the first substance into cells.

In one embodiment of the present invention the composition is comprised of 4 parts methanol to 1 part DMSO.

In another embodiment of the present invention the composition is comprised of 2.5 parts methanol, 2.5 parts ethanol, and 5 parts DMSO.

In yet another embodiment of the present invention the composition is comprised of 4 parts ethanol and 1 part DMSO.

In a preferred embodiment of the present invention, the composition is comprised of 1 part methanol and 1 part DMSO.

5 In another embodiment of the present invention, the composition is comprised of a single substance which can perform both functions of precipitating or denaturing proteins, and aiding in the infusion of the substance into cells.

In a preferred embodiment of the present invention, the composition is comprised of methanol only.

10 In another preferred embodiment of the present invention, the composition is comprised of dimethyl sulfoxide only.

### BRIEF DESCRIPTION OF THE DRAWING

15 FIG. 1 (A-E) is a schematic description of the Affirm VPIII nucleic acid hybridization assay.

20 FIG. 2A is a micrograph showing untreated *T. vaginalis* cells as compared to FIG. 2B showing a micrograph of *T. vaginalis* cells treated with a composition of the present invention.

25 FIG. 3A is a micrograph showing untreated *C. albicans* cells as compared to FIG. 3B showing a micrograph of *C. albicans* cells treated with a composition of the present invention.

30 FIG. 4A is a micrograph showing untreated human epidermal keratinocytes as compared to FIG. 4B showing a micrograph of human epidermal keratinocytes treated with a composition of the present invention.

FIG. 5A is a micrograph showing untreated *Spodoptera frugiperda* ovarian cells as compared to FIG. 5B showing a micrograph of *Spodoptera frugiperda* ovarian cells treated with a composition of the present invention.

35 FIG. 6A is a micrograph showing untreated human buccal cells as compared to FIG. 6B showing a micrograph of human buccal cells treated with a composition of the present invention.

### DETAILED DESCRIPTION OF THE INVENTION

40 The present invention provides a novel fixative composition and method for preserving cells in a sample, preferably a biological sample.

The composition of the present invention is comprised of:



- I. a first substance capable of precipitating or denaturing proteins, comprised of at least one alcohol or ketone; and
- II. a second facilitator substance to aid in the infusion of the first substance into cells.

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The alcohol or ketone may be one or more of the following: methanol, ethanol, propanol, isopropanol, butanol or acetone. The facilitator substance can be dimethyl sulfoxide, ethylene glycol, polyethylene glycol, or others familiar to those skilled in the art.

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There are several preferred embodiments of the composition of the present invention. The composition can be comprised of methanol:DMSO in a 4:1 ratio; methanol:ethanol:DMSO in a 2.5:2.5:5.0 ratio; ethanol:DMSO in a 4:1 ratio; or most preferably, methanol:DMSO in a 1:1 ratio. Other preferred embodiments include where the composition is comprised of methanol only or dimethyl sulfoxide only.

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In one preferred embodiment, the method of the present invention is directed to stabilizing the structure and nucleic acids of at least one cell in a sample, wherein said method comprises:

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(a) adding to a vessel containing the sample, a composition comprising an effective concentration of a substance capable of precipitating or denaturing proteins and capable of aiding in the infusion of said compound into said at least one cell;

(b) contacting said at least one cell in said sample with said composition;

25

(c) incubating said sample with said composition for an effective period of time and at an effective temperature; and

(d) obtaining said at least one cell with stabilized structure and nucleic acids in said sample.

30

The substance can be methanol, ethanol, propanol, isopropanol, butanol, acetone, dimethyl sulfoxide, ethylene glycol and polyethylene glycol.

In another preferred embodiment, the present invention relates to a method for stabilizing the structure and nucleic acids of at least one cell in a sample, wherein said method comprises:

35

(a) adding to a vessel containing the sample, a composition comprising an effective concentration of:

(i) a first substance capable of precipitating or denaturing proteins, comprising at least one alcohol or ketone; and

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(ii) a second facilitator substance to aid in the infusion of the first compound into the at least one cell;

(b) contacting said at least one cell in said sample with said composition;

(c) incubating said sample with said composition for an effective period of time and at an effective temperature; and

45

(d) obtaining said at least one cell with stabilized structure and nucleic acids in said sample.

The alcohol or ketone may be one or more of the following: methanol, ethanol, propanol, isopropanol, butanol or acetone. The facilitator substance can be dimethyl sulfoxide, ethylene glycol, polyethylene glycol or others familiar to those skilled in the art.

Specifically, vaginal swab samples fixed with the invention are stable for up to four days at ambient temperature and above, when stability is assessed as detectable *Trichomonas*, *Gardnerella* or *Candida* ribosomal RNA.

In a preferred embodiment, the invention can be used with the Affirm VPIII nucleic acid hybridization assay, described in U.S. Patent No. 5,654,418. However, the present invention is also intended for use with other test methods that rely on the integrity of nucleic acid for diagnosis.

Since the present invention preserves nucleic acid, and in a particularly preferred embodiment, RNA, in a prokaryotic cell type including, but not limited to, *Gardnerella vaginalis* (a Gram-negative bacterium) and a eukaryotic cell type, including but not limited to *Candida albicans* (a yeast) and *Trichomonas vaginalis* (a protozoan), it would be obvious to those skilled in the art that the preserving effects of the present solution can be applied to any other prokaryotic and eukaryotic cells, and is intended to be applicable to any other prokaryotic and eukaryotic cells.

In the preferred embodiment, vaginal swabs are placed in the Affirm VPIII tube, and a volume of the novel fixative (50 to 500 uls, preferably 100 uls), is added to the tube. Sample tubes are capped, and the samples can then be transported at ambient temperature to the site of testing. Testing can take place up to four days later. When ready for testing, a lysis solution is added to the tube, and the tube is capped and incubated at 85°C, which causes the organism to lyse and release nucleic acid (Figure 1A). After lysis and cooling, a buffer solution containing oligonucleotide probe polymers specific for 16s ribosomal ribonucleic acid (rRNA) of *G. vaginalis* and *C. albicans* is then added, forming a complex (Figure 1B). The polymer is not required for *T. vaginalis* because of the higher copy number of rRNA transcripts. The complex is then removed from the sample by a capture oligonucleotide that hybridizes to another site on the rRNA. There are three different capture oligonucleotides, each having unique specificity for *T. vaginalis*, *G. vaginalis* or *C. albicans*. Nylon beads are derivatized with only one type of capture oligonucleotide. One bead of each type is incubated with the sample, allowing for the simultaneous capture of rRNA of three distinct organisms (Figure 1C). The beads are next incubated in a solution containing biotinylated detector oligonucleotide probes. The detector probes hybridize to another region in the rRNA (Figure 1D). After washing, the bead is transferred to a well containing streptavidin conjugated to horseradish peroxidase (SA-HRP). If biotinylated detector probes are hybridized to the rRNA, the SA-HRP will bind to the biotin (Figure 1E). If there is no rRNA hybridized to the bead, no biotinylated detector probe will be present for the SA-HRP to bind. Finally the beads

are incubated with a substrate which will react with the HRP to form a blue color. If rRNA is present the beads will appear blue; if it is not present the beads remain colorless.

Alternative detection reagents can be used for research purposes to provide a quantitative measure of the relative amount of rRNA hybridized to the bead. After binding SA-HRP, beads can be incubated with a chemiluminescent HRP substrate in microtitre wells, and read in a luminometer.

In order to be effective, the fixative solution needs to inhibit or shut down the nucleases that are present in vaginal fluid matrix. Secondly, it has to permeate the organism and prevent nucleic acid degradation due to endogenous nucleases. Thirdly, it must leave the organism cell wall intact. Premature lysis of the organisms can result in a loss of some RNA when the sample is heated with the lysis solution in the Affirm VPIII assay. Fourthly, the fixative solution must be compatible with the Affirm VPIII reagents, not producing any artifact.

Many fixatives used for tissue fixation, staining and cryopreservation are complex solutions, often containing harsh reagents, with very limited stability. This is often necessary when the preservation of cellular structures or proteins is critical. Because the intent here is to preserve only the nucleic acid and cell wall, it is considered that the solution can be much less complex. An added benefit of the reduced complexity is a more stable solution.

Here it is described that mixtures of alcohol(s) and DMSO or these compounds alone are capable of stabilizing specimens, specifically vaginal swabs inoculated with causative agents for vaginitis and bacterial vaginosis. The novel solutions can serve as transport media for clinical specimens, which may be collected at remote sites and tested later at a centrally located processing laboratory. No special transport considerations are necessary, beyond careful handling and avoidance of temperature extremes.

The following are specific examples of the present invention, but should not in any way be considered limitations thereof.

#### **EXAMPLE 1.**

##### **Description of the Affirm VPIII Microbial Identification Test**

A description of the Affirm VPIII Microbial Identification Test and the Test protocol are as follows:

Collected vaginal swab samples are placed in the Affirm VPIII sample tube, and the swab shaft is snapped off at the score (just above the top of the tube). Twelve drops of Lysis Solution are added to each tube (with swabs remaining in the tubes), and the tubes are capped and placed in the Affirm Lysis block (at 85°C) for ten minutes. The

tube caps "capture" the swab shaft to facilitate later removal of the swab. After ten minutes, the tubes are removed from the block, and twelve drops of Buffer solution are added to each tube. The Buffer Solution contains the signal amplification polymers for *C. albicans* and *G. vaginalis*. Tubes are mixed by flicking briskly 10 times. The swabs  
 5 are then expressed on the sides of the tube to remove fluid, and discarded. The tube cap is replaced with a filter tip. Samples processed in this manner must be assayed within twenty-four hours.

The Affirm VPIII Reagent Cassette has seven wells, six of which contain reagents. One Reagent Cassette is used per sample. Well 1 is empty, and the prepared  
 10 sample is added to this well. Well 2 contains a Hybridization solution comprised of the biotinylated detector probes and formamide in a buffered chaotropic solution. Well 3 contains a Wash Solution. Well 4 contains the streptavidin-horseradish peroxidase (SA-HRP) conjugate. Wells 5 and 6 both contain Wash Solution. Well 7 contains a buffered  
 15 peroxide solution. Prior to running the test, four drops of Substrate Solution are added to Well 7. The substrate solution contains the indicator that reacts with the HRP to form the blue color, which is read visually. If a chemiluminescent indicator is used (for research purposes only), the Affirm Processor is stopped before the Probe Analysis Card (PAC) enters Well 7.

The Affirm VPIII Probe Analysis Card contains the three analyte-specific capture  
 20 beads previously described. In addition, there is one Negative control bead and one Positive control bead. One PAC is used per sample.

Affirm VPIII Reagent Cassettes are opened and arranged on the Affirm Automated Processor, one cassette per sample. The sample tubes are dispensed into well  
 25 1 of the Reagent Cassette. If the colorimetric, or visual detection method is used, four drops of Substrate Solution are added to Well 7. If a chemiluminescent detection method is to be used (for research purposes), then Substrate Solution is not added to Well 7. PACs are placed in well 1 of the Reagent Cassettes and the Processor is started.

The Processor arm picks up the PAC and gently agitates the card in the wells using a vertical, up-down motion. After several minutes in Well 1, the processor moves the PAC to well 2. If rRNA is present in the capture beads, the biotinylated detector  
 30 probes in well 2 will hybridize to the bead-RNA complex. The Positive control bead is derivatized with a capture oligonucleotide that is complementary to one of the detector oligonucleotides in Well 7. This ensures that the positive control bead is always positive,  
 35 unless the assay fails at the detection step. If the Positive control fails, it is a good indication that either 1) the user did not add the Substrate solution to Well 7, or 2) that there was a reagent failure in the cassette. Conversely, the Negative control bead is derivatized with an oligonucleotide that does not have a complementary detector  
 40 oligonucleotide in any of the wells. Therefore, this bead should always remain colorless. After Well 2, the Processor moves the PAC to Well 3, where Wash Solution removes any non-specifically bound nucleic acid or oligonucleotide probes. In Well 4, the biotinylated

detector probes bind to the SA-HRP conjugate. The PAC is then washed in two successive Wells, 5 and 6. This ensures that there will be no non-specific color development due to carryover of horseradish peroxidase. At this point the bead/nucleic-acid/detector/SA-HRP complex can be removed for chemiluminescent detection, or

5 allowed to proceed to Well 7 for reaction with the Indicator substrate. The PAC is then briefly washed in Well 6, and the assay is completed. Any blue color development on the specific analyte beads indicates that the sample contains that organism. A colorless specific analyte bead indicates that the sample is negative for that organism.

- 10 Samples were processed and tested according to the Affirm VPIII Microbial Identification Test protocol unless otherwise noted.

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## EXAMPLE 2.

### Description of the Experimental Procedure for Evaluating Preservative Solutions

Pools of vaginal fluid were prepared from fresh, self-collected vaginal swabs and/or frozen vaginal swabs. Fresh swab donors were instructed to place their swabs (5 per donor) on ice within 2 hours of collection. Swabs were kept on ice until pools were prepared, within two hours, or they were frozen immediately for later use. To prepare the vaginal pools, ten fresh or thawed frozen swabs were expressed per milliliter of Normal Saline (Cat. No. 4397753, Becton Dickinson Microbiology Systems, Sparks Maryland). Swabs were prepared in this manner until enough volume was obtained for the number of experimental samples being tested. A portion of the pool was used to resuspend organism pellets, as described below, and a portion was left uninoculated, to serve as the control.

*Candida albicans* (ATCC 60193) was grown on Sabouraud Dextrose agar (Cat. No. 4321278, Becton Dickinson Microbiology Systems, Sparks Maryland) at 35°C for 24-48 hours. A single colony was picked and streaked to a second plate, which was incubated for 18-20 hours at 35°C. After incubation, 3 mL of BSA/saline was added to the plate, and the colonies were gently resuspended with a 10uL inoculation loop. A sterile pipet was used to transfer the suspension to a tube containing at least 10mL of BSA/saline. The suspension was vortexed and measured spectrophotometrically for absorbance at 625nm. The suspension was adjusted to an OD<sub>625</sub> of approximately 0.4 (approximately  $6.0 \times 10^6$  CFU/mL). A volume of the suspension was then centrifuged for 10 minutes at 3000 rpm in a TJ-6 tabletop centrifuge (Beckman Instruments). The supernatant was decanted and the pellet was resuspended with the vaginal fluid to achieve a final concentration of approximately  $7.5 \times 10^6$  CFU/mL.

*Gardnerella vaginalis* (ATCC 49145) was grown on Chocolate II agar (Cat. No. 4321267, Becton Dickinson) at 35°C for 24-48 hours. Single colonies were picked and streaked to secondary plates, which were incubated for 18-48 hours at 35°C. After incubation, 3 mL of BSA/saline was added to each secondary plate, and the colonies were gently resuspended with a 10uL inoculation loop. A sterile pipet was used to transfer the suspension to a tube containing at least 10mL of BSA/saline. The suspension was vortexed and measured spectrophotometrically for absorbance at 625nm. The suspension was adjusted to an OD<sub>625</sub> of approximately 0.3 (approximately  $2.5 \times 10^8$  CFU/mL). A volume of the suspension was then centrifuged for 10 minutes at 3000 rpm in a TJ-6 tabletop centrifuge (Beckman Instruments). The supernatant was decanted and the pellet was resuspended with the vaginal fluid to achieve a final concentration of approximately  $2.5 \times 10^9$  CFU/mL.

*Trichomonas vaginalis* (ATCC 30001) was grown in 10mL tubes of Modified Diamond's Medium (Cat. No. 07-097, Remel ) at 37°C in 5-10% CO<sub>2</sub> for five days or until it reached a density of approximately  $2 \times 10^5$  cells/mL. Secondary cultures were prepared from cultures that contained  $1 \times 10^5$  to  $1.5 \times 10^6$  Trichomonads/mL and were free

of contamination. Fresh, pre-warmed tubes of medium were inoculated with 0.5 to 1.0 ml of the culture. Tubes were capped loosely and incubated for 2 to 3 days at 37°C in 5-10% CO<sub>2</sub>. Secondary cultures that were at least 2x10<sup>5</sup> Trichomonads/mL were pooled, mixed and counted using a hemacytometer. The suspension was spun for seven minutes  
 5 at 2000 rpm in a TJ-6 tabletop centrifuge. The supernatant was decanted and the pellet was resuspended with the vaginal fluid to achieve a final concentration of approximately 6 x 10<sup>6</sup> Trichomonads/mL.

Typically, all three organisms were tested in an experiment. In this case, the  
 10 pellets of each organism, *C. albicans*, *G. vaginalis* and *T. vaginalis* were sequentially resuspended with the same vaginal fluid. Serial ten-fold dilutions of the spiked vaginal fluid were plated in duplicate on Chocolate II agar plates to determine actual viable cfu/mL concentrations of *Gardnerella vaginalis* and *Candida albicans*.

15 Glass screw capped tubes, or Affirm VPIII sample tubes were prepared by pipetting 100 uL of the preservative/fixative solution under test into the tubes. Nothing was pipetted into negative control (no preservative) tubes. Three replicates were prepared for each test condition and each time point. Affirm Sample Collection swabs (Cat. No. 4406251) were seeded with 100ul of vaginal fluid, or spiked vaginal fluid. Swabs were  
 20 then placed into the glass or Affirm VPIII tubes and capped. Initial time point samples, *t*<sub>0</sub>, were processed immediately. The remaining samples were processed at approximately 24, 48, 72 or 96 hours later. Testing proceeded according to the protocol described in Example 1.

25 For quantitative detection, a chemiluminescent substrate was used. The detection substrate was lumino/4-iodophenol (Boehringer-Mannheim Chemiluminescence ELISA Substrate, catalog no. 1582950). Briefly, PACs were removed from the Affirm Processor just prior to entering Well 7. Capture beads were removed for the PACs and placed into white, flat-bottomed microtiter wells, one bead per well. The recipient wells contained  
 30 100ul of Wash Solution, to keep the beads from drying out. Once the plate was full, the Wash Solution was aspirated from the wells using a multi-channel pipet, and 100ul of the chemiluminescent substrate solution was added per well. The plate was immediately placed on a plate reader (Dynatek ML3000, internal temperature 30°C, 7 cycles, 61 second pause between cycles) and read for 7 cycles. Data from the 5<sup>th</sup> cycle  
 35 (approximately ten minutes after the addition of substrate) was used for all analyses.

40

**EXAMPLE 3.****Common Fixatives and Preservatives Tested for Usefulness with Affirm Samples**

- 5 The following table lists a number of commercially available preservatives and fixatives or solutions prepared in-house using standard formulations. These solutions were tested in the Affirm VPIII Microbial Identification Test with samples prepared according to the protocol described in EXAMPLE 2, using glass sample tubes and chemiluminescent detection. Solutions that failed to provide at least 24 hours of sample
- 10 stability are designated as "FAILED", while solutions that interfered with the Affirm assay are designated as "INTERFERED".

Reagent	Manufacturer	Status
PreservCyt (50% Methanol, Acetic Acid, EDTA)	Cytec	FAILED
95% Methanol, Acetic Acid, EDTA	Prep'd in house	FAILED
95% Methanol, Acetic Acid, EDTA, Lithium Chloride	Prep'd in house	FAILED
95% Methanol, Acetic Acid	Prep'd in house	FAILED
95% Methanol, EDTA	Prep'd in house	FAILED
95% Methanol	Prep'd in house	FAILED
Buffered Formalin	SDL Inc.	FAILED
Parasafe	SDL Inc.	FAILED
PVA (low density poly vinyl alcohol)	SDL Inc.	Interfered
Affirm VPIII Buffer	Prep'd in house	FAILED
Modified Affirm Buffer (0.5x, with Lithium Chloride)	Prep'd in house	FAILED
Carnoy's solution (60% ethanol, 30% chloroform, 10% acetic acid)	Prep'd in house	Interfered
Acid/Ethanol (80% ethanol, 20% acetic acid)	Prep'd in house	FAILED
PACE Transport Buffer	Gen-Probe	FAILED
RNA Later	Ambion	FAILED
Molecular Biology Fixative	Streck Labs.	FAILED
CytoRich Blue Preservative Fluid	AutoCyte, Inc	FAILED



**EXAMPLE 4.****Preservative Effects of Three Formulations Over Twenty –Four Hours**

Three formulations of methanol and dimethyl sulfoxide solution were prepared and tested for their preservative ability over twenty four hours with *C. albicans*, *G. vaginalis* and *T. vaginalis*. The procedure was as described in Example 2, using glass sample tubes. Organism cfu per ml of vaginal fluid were as follows: *Trichomonas*,  $6 \times 10^6$ /mL; *Gardnerella*,  $1.5 \times 10^9$ /mL; *Candida*,  $1 \times 10^7$ /mL. Signal was measured using chemiluminescent detection methods, as described in Example 2. Reported values (Relative Luminescence Units, RLUs) are the mean of three replicates.

Solution	Hours	Negative	Positive	Tv	Gv	Ca
None	0	0.0	6.8	5.3	154.4	11.3
	24	0.5	9.7	0.1	77.5	2286.9
80% Methanol, 20% DMSO	0	0.0	9.8	5.9	103.8	15.8
	24	0.0	9.3	6.4	102.0	15.3
50% Methanol, 50% DMSO	0	0.1	10.3	5.8	171.5	22.4
	24	0.0	9.7	5.9	196.1	15.1
100% Methanol	0	0.0	10.6	7.0	172.7	16.7
	24	0.0	9.8	7.1	106.9	16.4

The data demonstrated that with no preservative solution, *T. vaginalis* signal was negative, *G. vaginalis* signal sharply declined, and *C. albicans* signal sharply increased (due to growth) by 24 hours. With the preservative solutions, signals remained relatively constant over 24 hours of ambient temperature storage of the samples.

**EXAMPLE 5.****Preservative Effect of Additional Formulations Over Twenty-Four Hours**

- 5 Additional formulations of preservative solution were tested for their preservative ability over twenty four hours with *C. albicans* and *G. vaginalis*. The procedure was as described in Example 2, using glass sample tubes. Organism cfu per ml of vaginal fluid were as follows: *Gardnerella*,  $9.7 \times 10^8$ /mL; *Candida*,  $3.4 \times 10^6$ /mL. Signal was measured using chemiluminescent detection methods, as described in Example 2. Reported values (Relative Luminescence Units, RLUs) are the mean of three replicates.

10

Solution	Hours	Negative	Positive	Gv	Ca
None	0	0.0	8.2	140.4	15.7
	24	0.0	10.3	71.0	1721.1
40% Methanol, 40% Ethanol, 20% DMSO	0	0.0	8.9	52.3	10.4
	24	0.0	9.8	95.2	12.1
25% Methanol, 25% Ethanol, 50% DMSO	0	0.0	10.0	124.0	16.8
	24	0.0	9.7	165.7	15.8
80% Ethanol, 20% DMSO	0	0.1	9.9	127.2	16.1
	24	0.0	10.2	102.2	13.1

- 15 The data indicated that with no preservative solution *G. vaginalis* signal sharply declined, and *C. albicans* signal sharply increased (due to growth) by 24 hours. With the preservative solutions, signals remained relatively constant over 24 hours of ambient temperature storage of the samples.

## EXAMPLE 6

## Sample Stability at Forty-Eight Hours with Several Formulations

- Five preservative formulations were tested for their preservative ability over forty eight hours with *T. vaginalis*, *C. albicans* and *G. vaginalis*. The procedure was as described in Example 2, using glass sample tubes. Organism cfu per ml of vaginal fluid were as follows: *Trichomonas*,  $4.7 \times 10^7$ /mL; *Gardnerella*,  $1.4 \times 10^8$ /mL; *Candida*,  $5.6 \times 10^6$ /mL. Signal was measured using chemiluminescent detection methods, as described in Example 2. Reported values (Relative Luminescence Units, RLUs) are the mean of three replicates.

Solution	Hours	Negative	Positive	Tv	Gv	Ca
None	0	0.0	9.5	87.0	190.0	16.8
	24	0.0	8.1	9.1	16.7	1528.9
	48	0.0	9.6	0.6	1.7	1394.9
80% Methanol, 20% DMSO	0	0.0	10.2	68.4	70.1	8.1
	24	0.0	9.4	66.3	171.6	4.8
	48	0.0	8.8	75.3	127.4	6.8
50% Methanol, 50% DMSO	0	0.1	8.8	68.7	79.6	7.5
	24	0.0	11.0	67.7	162.1	5.9
	48	0.0	8.4	79.5	174.9	5.7
40% Methanol, 40% Ethanol, 20% DMSO	0	0.0	8.1	87.6	179.4	7.8
	24	0.0	10.0	75.3	136.2	4.8
	48	0.0	8.4	70.7	75.5	5.4
25% Methanol, 25% Ethanol, 50% DMSO	0	0.0	9.0	96.2	228.8	7.9
	24	0.0	8.1	67.4	132.7	2.7
	48	0.0	8.4	70.8	124.6	5.0
80% Ethanol, 20% DMSO	0	0.0	9.1	97.4	128.0	8.2
	24	0.0	9.4	66.1	117.2	2.7
	48	0.0	8.9	70.2	84.8	5.4

- The data indicated that with no preservative solution *T. vaginalis* and *G. vaginalis* signals were negative at 48 hours, and *C. albicans* signal was sharply increased (due to growth) by 24 and 48 hours. With the preservative solutions, signals remained relatively constant over 48 hours of ambient temperature storage of the samples.

**EXAMPLE 7.****Sample Stability with Preservative in the Affirm VPIII Sample Tube**

- One preservative formulation was tested over forty eight hours with *T. vaginalis*,  
 5 *C. albicans* and *G. vaginalis*, using either glass screw capped tubes or the low density  
 polyethylene Affirm VPIII processing tube. The procedure was as described in Example  
 2. Organism cfu per ml of vaginal fluid were as follows: *Trichomonas*,  $3.9 \times 10^7$ /mL;  
*Gardnerella*,  $3.1 \times 10^8$ /mL; *Candida*,  $5.3 \times 10^6$ /mL. Signal was measured using  
 chemiluminescent detection methods, as described in Example 2. Reported values  
 10 (Relative Luminescence Units, RLUs) are the mean of three replicates.

Solution	Tube	Hours	Negative	Positive	Tv	Gv	Ca
None	Glass	0	0.0	8.5	47.5	164.5	7.9
		24	0.0	9.7	1.2	20.1	1093.0
		48	0.0	10.9	0.1	.8	1583.0
	Affirm	0	0.0	7.8	85.4	204.1	13.1
		24	0.0	9.5	8.3	98.6	857.3
		48	0.0	10.2	3.0	1.5	1152.5
50% Methanol, 50% DMSO	Glass	0	0.0	8.7	43.9	202.7	6.2
		24	0.0	9.9	41.9	292.7	4.1
		48	0.0	9.3	39.5	296.4	5.8
	Affirm	0	0.0	8.5	119.3	476.7	4.4
		24	0.2	10.1	109.4	725.7	5.4
		48	0.0	11.7	116.5	890.5	5.0

- The results indicated that the Affirm VPIII sample tube offered stability  
 15 comparable to the glass screw capped tubes when preservative solution was used. In fact,  
 less sample was lost using the Affirm VPIII tube because the swab was transported and  
 processed in the same tube. Swabs transported in glass tubes were transferred to Affirm  
 VPIII tubes for processing. Neither tube maintained sample stability when no  
 preservative solution was used.

20

**EXAMPLE 8.****Solution Performance Across Sample Matrix Pools**

- An experiment was performed to demonstrate the preservative solution  
 25 performance across different vaginal fluid matrices. Four pools of vaginal fluid were  
 prepared: two from freshly collected swabs and two from frozen swabs. Affirm VPIII  
 tubes were used for sample transport conditions. The procedure was as described in  
 Example 2. The average organism cfu per ml of vaginal fluid was as follows:  
*Trichomonas*,  $5 \times 10^7$ /mL; *Gardnerella*,  $2.1 \times 10^8$ /mL; *Candida*,  $5.6 \times 10^7$ /mL. Signal was

measured using chemiluminescent detection methods, as described in Example 2. Reported values (Relative Luminescence Units, RLUs) are the mean of three replicates.

Vaginal pool	Solution	Hours	Negative	Positive	Tv	Gv	Ca
Pool 1 (frozen)	None	0	0.0	7.5	83.3	141.5	27.5
		24	0.1	12.9	14.2	78.7	1255.8
		48	0.0	8.9	1.9	4.4	751.2
		72	0.1	18.8	0.8	7.7	2619.2
	50% MeOH, 50% DMSO	0	0.0	8.8	68.1	245.7	12.1
		24	0.0	10.5	58.9	249.4	24.3
		48	0.0	9.4	56.1	299.9	13.5
		72	0.0	8.1	31.1	222.3	20.2
Pool 2 (frozen)	None	0	0.0	7.6	93.8	89.0	20.8
		24	0.0	9.7	7.8	32.7	1316.3
		48	0.0	7.3	1.5	1.8	955.4
		72	0.0	9.6	0.2	2.4	1773.7
	50% MeOH, 50% DMSO	0	0.0	6.7	99.3	168.7	9.2
		24	0.1	11.0	94.2	168.2	13.5
		48	0.0	9.1	76.5	143.3	9.9
		72	0.0	9.3	60.2	221.3	11.1
Pool 3 (fresh)	None	0	0.0	8.6	86.9	76.3	17.1
		24	0.0	9.2	0.0	8.4	1244.7
		48	0.0	9.6	0.0	2.6	1135.8
		72	0.0	8.9	0.0	0.9	1205.1
	50% MeOH, 50% DMSO	0	0.0	5.6	79.2	118.6	6.1
		24	0.2	9.2	95.7	155.8	11.6
		48	0.0	9.2	62.8	107.8	6.4
		72	0.0	7.5	54.4	159.4	9.2
Pool 4 (fresh)	None	0	0.0	8.2	68.9	119.6	18.5
		24	0.0	7.9	0.7	54.4	1226.7
		48	0.0	8.8	0.0	11.8	1086.9
		72	0.0	6.0	0.0	2.6	1223.4
	50% MeOH, 50% DMSO	0	0.0	9.5	94.0	172.6	11.2
		24	0.0	8.3	44.9	144.0	9.6
		48	0.0	6.3	45.9	135.9	5.1
		72	0.0	7.1	29.8	147.1	6.7

- 5 The results showed that the solution was capable of maintaining signal of the organisms in the four vaginal fluid pools over 72 hours of storage time.

**EXAMPLE 9.****Solution Performance Across Sample Matrix From Fifteen Individuals**

- 5 An experiment was performed to demonstrate the performance of the preservative solution (50% Methanol, 50% dimethyl sulfoxide) across vaginal fluid collected from fifteen women. Ten swabs were collected per day for two days from each donor. Affirm VPIII tubes were used for sample transport conditions. The procedure was as described in Example 2. The average organism cfu per ml of vaginal fluid was as follows:
- 10 *Trichomonas*,  $3.5 \times 10^7$ /mL; *Gardnerella*,  $6.0 \times 10^8$ /mL; *Candida*,  $7.7 \times 10^6$ /mL. Signal was measured using chemiluminescent detection methods, as described in Example 2. Reported values (Relative Luminescence Units, RLUs) are the mean of three replicates.

Donor	Hours	Negative	Positive	Tv	Gv	Ca
1	0	0.0	7.8	47.1	165.0	15.3
	24	0.0	7.6	67.5	277.3	12.7
	48	0.0	12.8	47.3	249.0	16.0
	72	0.0	7.8	43.7	214.6	9.4
2	0	0.0	5.4	67.6	254.4	15.0
	24	0.0	6.4	91.8	464.6	27.8
	48	0.0	11.1	99.7	665.2	27.7
	72	0.0	9.5	61.6	485.7	16.2
3	0	0.0	8.6	72.4	268.7	16.5
	24	0.0	9.1	65.7	308.0	19.7
	48	0.0	14.8	81.8	449.7	28.6
	72	0.0	8.2	49.4	301.1	16.7
4	0	0.0	8.9	65.3	206.1	15.6
	24	0.0	8.7	39.0	203.4	18.8
	48	0.0	15.2	40.0	261.5	29.4
	72	0.0	6.4	23.6	216.7	13.3
5	0	0.0	9.3	57.6	165.3	11.8
	24	0.0	8.6	50.8	266.5	12.8
	48	0.1	12.1	64.6	299.9	24.0
	72	0.0	8.5	44.6	259.4	11.8
6	0	0.0	8.5	63.3	464.7	16.3
	24	0.0	7.1	40.9	259.0	10.4
	48	0.0	8.6	49.7	349.5	12.4
	72	0.0	8.1	44.9	486.6	15.9
7	0	0.0	8.9	62.6	318.1	20.0
	24	0.0	8.2	44.7	244.9	9.5
	48	0.0	8.4	34.7	226.1	9.5
	72	0.0	8.9	33.5	284.1	12.9

Donor	Hours	Negative	Positive	Tv	Gv	Ca
8	0	0.0	8.1	60.2	291.6	11.2
	24	0.0	7.9	42.3	168.2	7.5
	48	0.0	9.8	44.4	190.9	7.1
	72	0.0	8.9	53.1	292.6	8.9
9	0	0.0	8.6	62.2	273.6	17.6
	24	0.0	8.1	27.2	191.7	12.6
	48	0.0	8.6	21.3	222.8	16.7
	72	0.0	12.0	29.1	379.5	16.7
10	0	0.0	8.3	39.8	145.7	12.2
	24	0.1	7.5	26.5	103.7	8.9
	48	0.2	9.9	22.9	128.0	11.0
	72	0.0	9.2	31.1	208.9	14.1
11	0	0.0	8.5	113.2	218.7	8.2
	24	0.0	8.1	95.6	156.6	5.1
	48	0.0	11.9	158.2	332.6	8.2
	72	0.0	8.6	90.8	178.2	3.0
12	0	0.0	9.5	112.4	189.7	11.7
	24	0.0	8.6	66.0	172.4	11.9
	48	0.0	13.2	114.6	346.8	22.0
	72	0.0	9.8	60.4	170.4	7.5
13	0	0.0	9.8	130.2	159.9	7.3
	24	0.0	10.0	118.5	191.4	10.2
	48	0.0	13.2	222.4	322.2	14.7
	72	0.0	9.0	125.0	225.3	7.8
14	0	0.0	8.6	118.7	138.7	9.7
	24	0.0	8.7	91.2	126.1	6.0
	48	0.0	15.2	160.6	213.9	12.3
	72	0.0	9.3	89.5	105.5	4.3
15	0	0.0	10.1	103.2	97.8	7.5
	24	0.0	8.7	88.0	123.5	11.9
	48	0.0	14.6	122.6	168.6	18.1
	72	0.0	9.3	97.8	117.7	10.8

The solution demonstrated preservative effects across the vaginal fluid from 15 different individual donors. All organism signals were maintained.

**EXAMPLE 10.****Solution Performance with Samples Stored at a Range of Temperatures**

- 5 An experiment was performed to demonstrate the performance of the preservative solution (50% Methanol, 50% dimethyl sulfoxide) across a range of storage temperatures. Ten swabs were collected per day for two days from each donor. Affirm VPIII tubes were used for sample transport conditions. The procedure was as described in Example 2. Organism cfu per ml of vaginal fluid were as follows: *Trichomonas*,  $8.2 \times 10^6$ /mL; *Gardnerella*,  $1.4 \times 10^7$ /mL; *Candida*,  $7.9 \times 10^6$ /mL. Signal was measured using
- 10 colorimetric detection methods, as described in Example 2, except the bead color was assigned a value relative to the positive control bead color. Positive control beads were assigned a "2". Analyte beads darker than the control bead were assigned a "3", analyte beads the same color as the control bead were assigned a "2", and analyte beads lighter than the control bead, but darker than the negative control bead, were assigned a "1".
- 15 Reported values are the mode of three replicates, each scored by three different readers. Samples were stored up to 96 hours.

Solution	Temperature	Hours	Negative	Positive	Tv	Gv	Ca
None	Ambient	0	0	2	3	2	3
		24	0	2	0	0	3
		48	0	2	0	0	3
		96	0	2	0	0	3
50% Methanol, 50% DMSO	Ambient	0	0	2	3	3	3
		24	0	2	3	3	3
		48	0	2	2	2	3
		96	0	2	2	3	3
	2°C	0	0	2	3	3	3
		24	0	2	3	3	3
		48	0	2	3	3	2
		96	0	2	3	3	2
	30°C	0	0	2	3	3	3
		24	0	2	2	2	2
		48	0	2	2	2	3
		96	0	2	1	1	1
	35°C	0	0	2	3	3	3
		24	0	2	2	3	2
		48	0	2	1	3	2
		96	0	2	2	3	3



The data showed that the solution was capable of providing sample stability at a range of storage temperatures over 96 hours. It also indicated that the preservative solution did not have an effect on the method of detection.

5

**EXAMPLE 11.****Demonstration That the Solution Provides Protection Against Nucleases**

10 The preferred embodiment (50% Methanol, 50% DMSO) was tested for its ability to protect RNA against nuclease degradation. A commercially available RNase detection kit (RNaseAlert™RNase Detection Dipsticks, catalog no 1960, Ambion) was used with samples of vaginal fluid alone and vaginal fluid with preservative solution added. The kit provides an RNA solution that is spotted onto a dipstick in two places, according to kit protocol. One spot is incubated with the test solution. Both spots on the dipstick are then subjected to colorimetric detection. If the RNA spot that was incubated with the test solution is white or a lighter shade of blue than the control spot, the test solution contains RNase. The strips were used to test vaginal fluid that had been mixed with the preservative solution. The results indicated that the vaginal fluid mixed with preservative solution left detectable RNA on the test spot more often than untreated vaginal fluid. This indicated that the preservative solution provided protection against nucleases present in the vaginal fluid.

25 In a separate experiment, the RNaseAlert™RNase Detection Dipsticks were used to assess the preservative solution with human whole blood, serum and plasma. Samples of each were mixed 1:1 with the preferred embodiment (50% methanol, 50% DMSO) and tested with the dipsticks. Samples containing no preservative were tested as well. The results indicated that the whole blood, serum and plasma samples contained active RNases, since no visible spot remained on the test strip after the protocol was completed. However, when whole blood, serum and plasma were mixed with the preservative solution, the test RNA spot was clearly visible on the strip. This demonstrated that the preservative solution provided protection against nucleases present in human whole blood, serum and plasma.

35

**EXAMPLE 12.**  
**Performance Over a Broad Range of Solution Compositions**

- 5 An experiment was performed to demonstrate the performance of the preservative solution when either component was varied to extreme concentrations. Samples were stored in the preservative for up to 72 hours. Affirm VPIII tubes were used for sample transport conditions. The procedure was as described in Example 2. Organism cfu per ml of vaginal fluid were as follows: *Trichomonas*,  $1 \times 10^7$ /mL; *Gardnerella*,  $1.9 \times 10^7$ /mL; *Candida*,  $1.4 \times 10^7$ /mL. Signal was measured using colorimetric detection methods, as
- 10 described in Example 10. Reported values are the average of three replicates, each scored by three different readers. Preservative effects were observed for all formulations tested.

Formulation	Hours	Negative	Positive	Tv	Gv	Ca
None	0	0	2	3	1	3
	24	0	2	1	0	4
	72	0	2	0	0	4
20% Methanol 80% DMSO	0	0	2	3	2	3
	24	0	2	3	2	3
	72	0	2	2.9	1.7	3
40% Methanol 60% DMSO	0	0	2	3	2	3
	24	0	2	3	2	3
	72	0	2	3	2.2	3
50% Methanol 50% DMSO	0	0	2	3	2	3
	24	0	2	3	2	3
	72	0	2	3	2	3
60% Methanol 40% DMSO	0	0	2	3	2	3
	24	0	2	3	2	3
	72	0	2	3	2	3
80% Methanol 20% DMSO	0	0	2	3	2	3
	24	0	2	3	2	3
	72	0	2	3	1.2	3
100% Methanol	0	0	2	3	2	3
	24	0	2	2.9	2	3
	72	0	2	3	1.8	3
100% DMSO	0	0	2	3	2	3
	24	0	2	3	2	3
	72	0	2	3	2	3

**EXAMPLE 13.****Microscopic Evaluation of Cells Treated with Preservative Solution**

Cells from various organisms were pelleted by centrifugation and then resuspended in BSA/saline. An aliquot of each cell suspension was treated with an equal volume of preservative solution comprised of a 1:1 mixture of methanol and DMSO. The treated cells were stored at ambient temperatures for 24 hours. As a control, a second aliquot of cells was treated with an equal volume of BSA/saline immediately before microscopic examination. Both aliquots were examined using a phase contrast microscope (Eclipse E400, Nikon Japan) under 40x power.

Cell	Origin	Results
<i>Trichomonas vaginalis</i>	ATCC 30001	Figures 2a and 2b
<i>Candida albicans</i>	ATCC 60193	Figures 3a and 3b
Human epidermal keratinocytes	Cell line NHEK-3007 (BioWhittaker Cat. No. CC-2503)	Figures 4a and 4b
<i>Spodoptera frugiperda</i> ovarian cells	Sf9 insect cell line (PharMingen Cat. No. 21300L)	Figures 5a and 5b
Human buccal cells	Cheek scrapings	Figures 6a and 6b

Microscopic examination revealed cells that were visually intact after 24 hours of ambient storage in the preservative solution. Cells were similar in appearance and number to the untreated controls. These results indicate that the solution does not lyse the cells, but stabilizes their structure and keeps the cells intact.

## WHAT IS CLAIMED IS:

1. A composition for stabilizing the structure and nucleic acids of at least one cell, said composition being comprised of:

- (a) a first substance capable of precipitating or denaturing proteins, comprising at least one alcohol or ketone; and
- (b) a second facilitator substance to aid in the infusion of the first substance into at least one cell,

wherein the concentrations of said first and second substances are effective to stabilize the structure and nucleic acids of said at least one cell.

2. The composition of Claim 1 wherein said at least one alcohol or ketone is selected from the group consisting of methanol, ethanol, propanol, isopropanol, butanol and acetone.

3. The composition of Claim 1 wherein said second substance is selected from the group consisting of dimethyl sulfoxide, ethylene glycol and polyethylene glycol.

4. The composition of Claim 1 wherein said first substance is comprised of one alcohol or ketone.

5. The composition of Claim 4 wherein said concentrations of said first and second substances in said composition are in a ratio of 4:1 (first substance : second substance).

6. The composition of Claim 1 wherein said first substance is comprised of a first alcohol or ketone and a second alcohol or ketone.

7. The composition of Claim 6 wherein said concentrations of said first and second substances in said composition are in a ratio of 2.5:2.5:5 (first alcohol or ketone : second alcohol or ketone : second substance).

8. The composition of Claim 4 wherein said concentrations of said first and second substances in said composition are in a ratio of 1:1 (first substance : second substance).

9. The composition of Claim 5 wherein said first substance is methanol and said second substance is dimethyl sulfoxide.

10. The composition of Claim 7 wherein said first alcohol or ketone is methanol, said second alcohol or ketone is ethanol and said second substance is dimethyl sulfoxide.

11. The composition of Claim 5 wherein said first substance is ethanol and said second substance is dimethyl sulfoxide.

12. The composition of Claim 8 wherein said first substance is methanol and said second substance is dimethyl sulfoxide.

13. The composition of Claim 1 wherein said nucleic acid is DNA.

14. The composition of Claim 1 wherein said nucleic acid is RNA.

15. The composition of Claim 14 wherein said RNA is ribosomal RNA.

16. The composition of Claim 1 wherein said at least one cell is a prokaryote or eukaryote.

17. The composition of Claim 1 wherein said at least one cell is a microorganism.

#24901

**ABSTRACT OF THE INVENTION**

The present invention relates to a method and composition for stabilizing clinical specimens (i.e., cells in biological samples) for transport and subsequent testing for diagnosis. The composition is specifically capable of maintaining nucleic acid in the cells intact for hybridization with oligonucleotide capture and detector probes.

#24777

FIG. 1

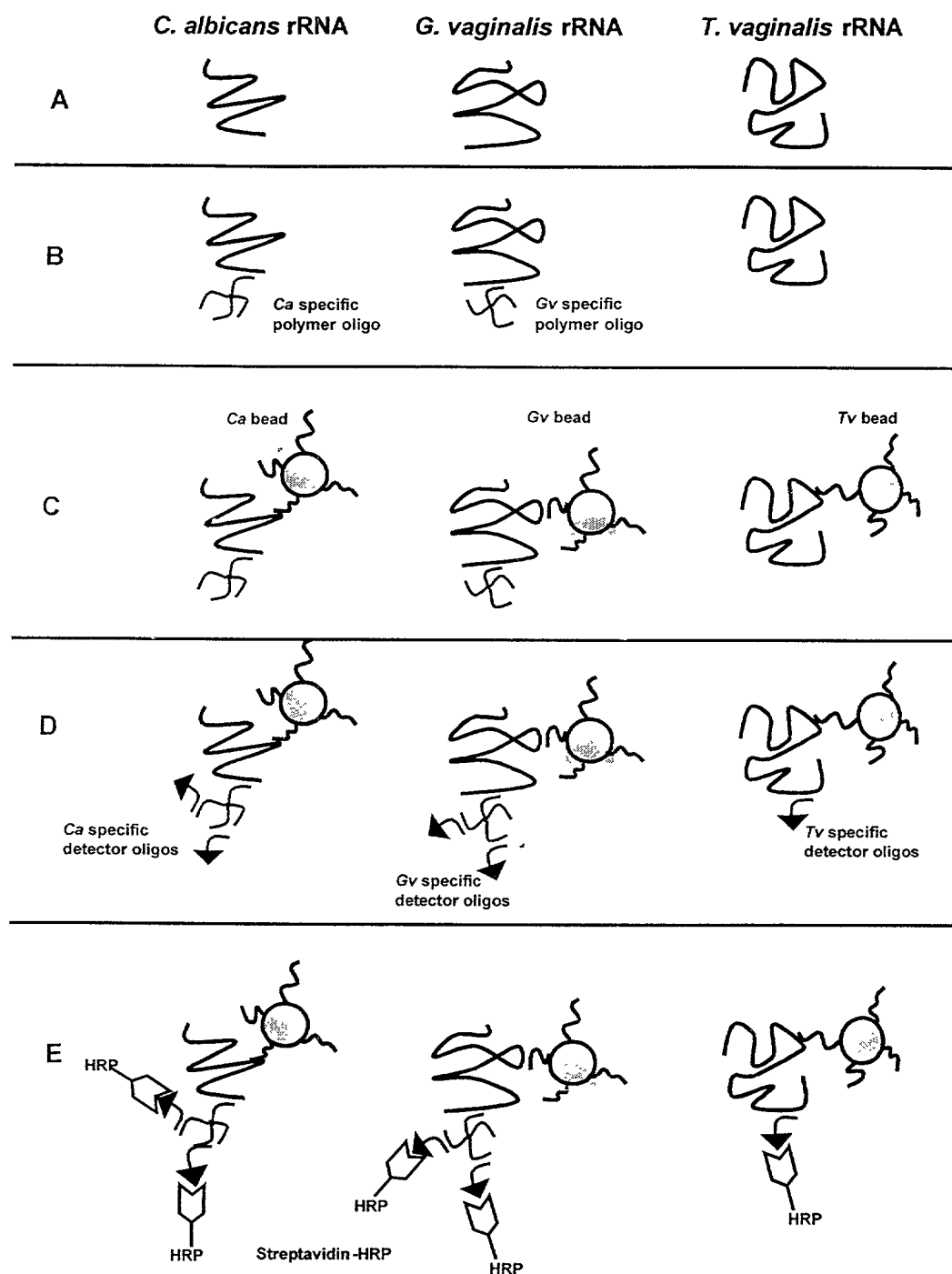


FIG. 2

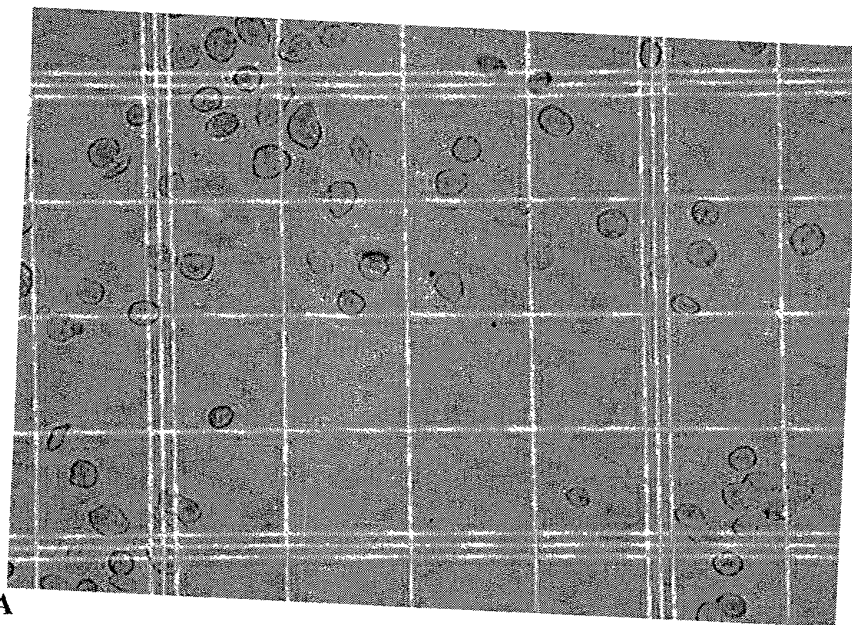


FIG. 2A

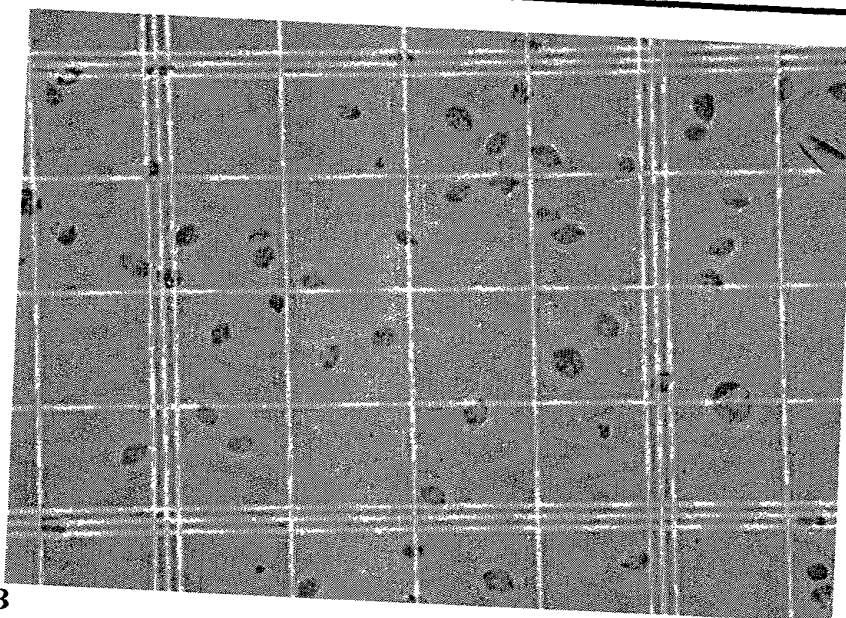
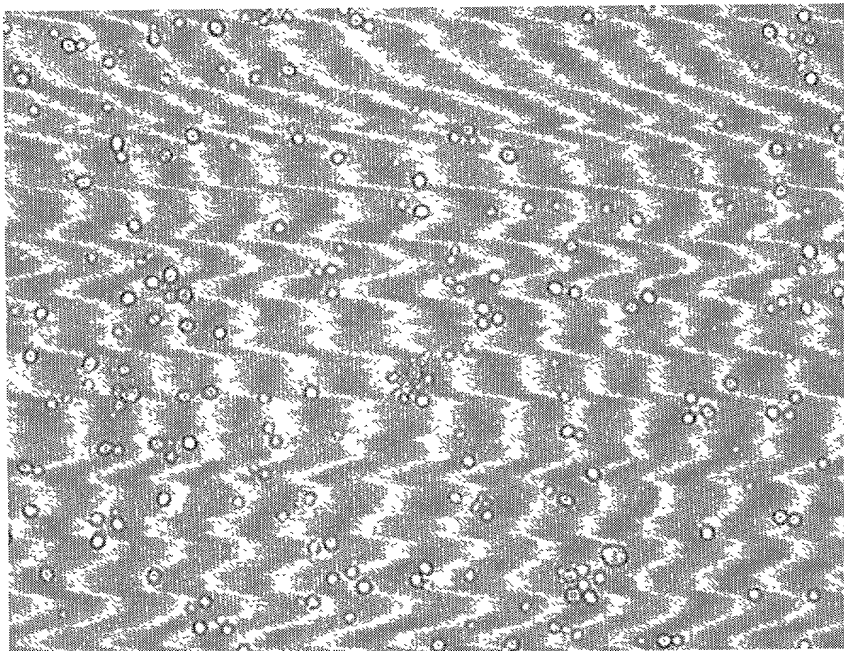


FIG. 2B

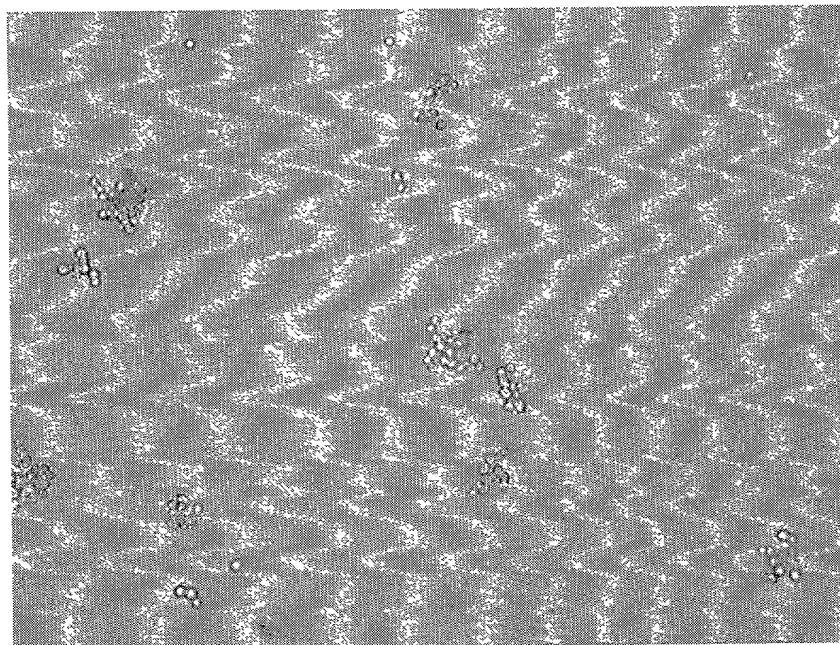
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**FIG. 3**



**FIG. 3A**



**FIG. 3B**

FIG. 4

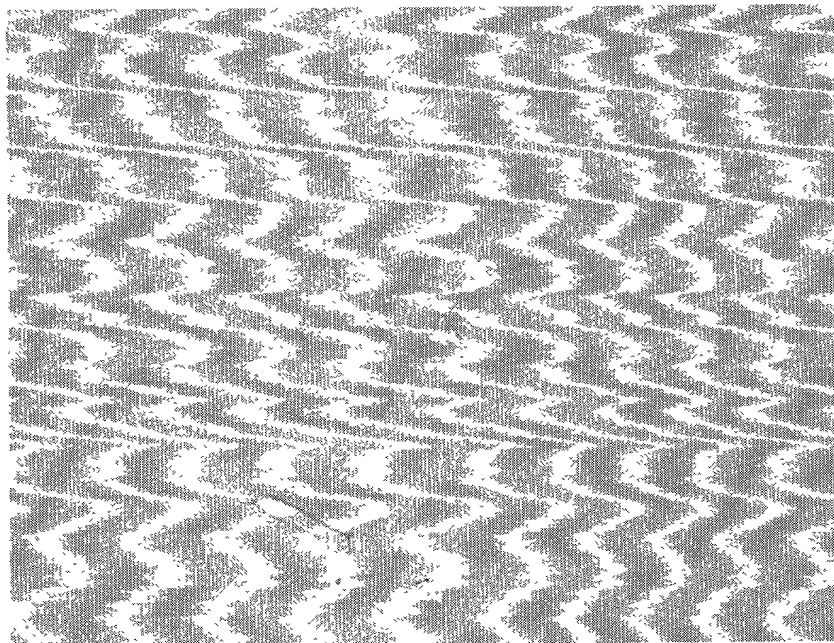


FIG. 4A

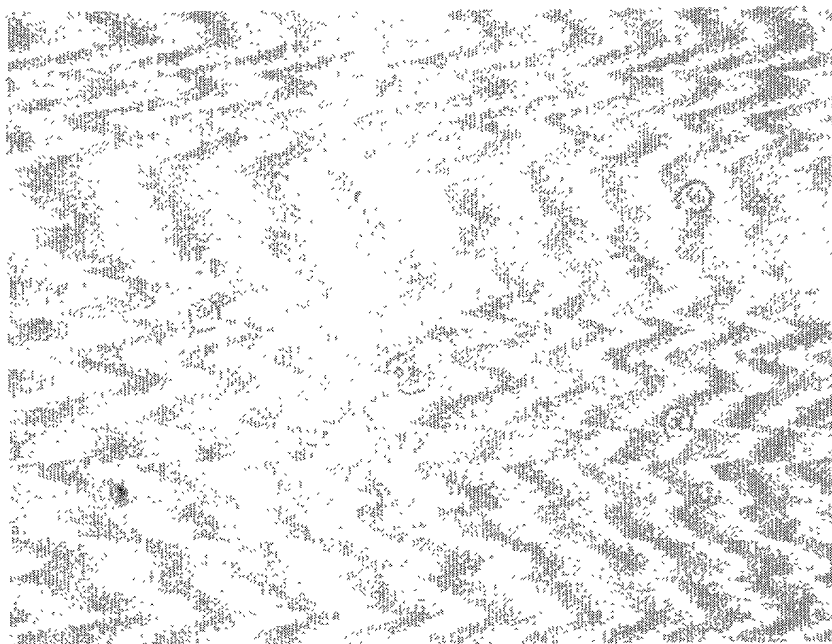


FIG. 4B

FIG. 5

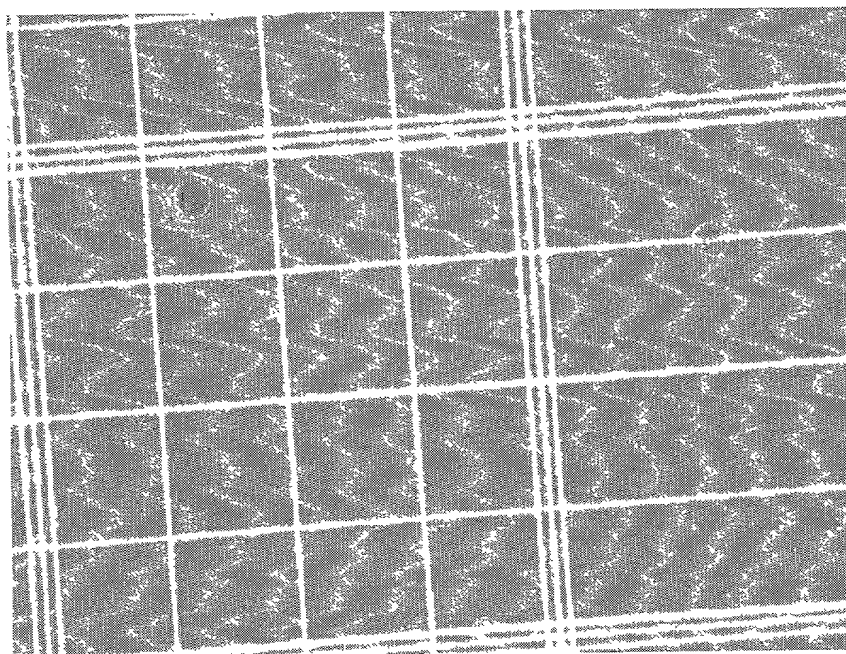


FIG. 5A

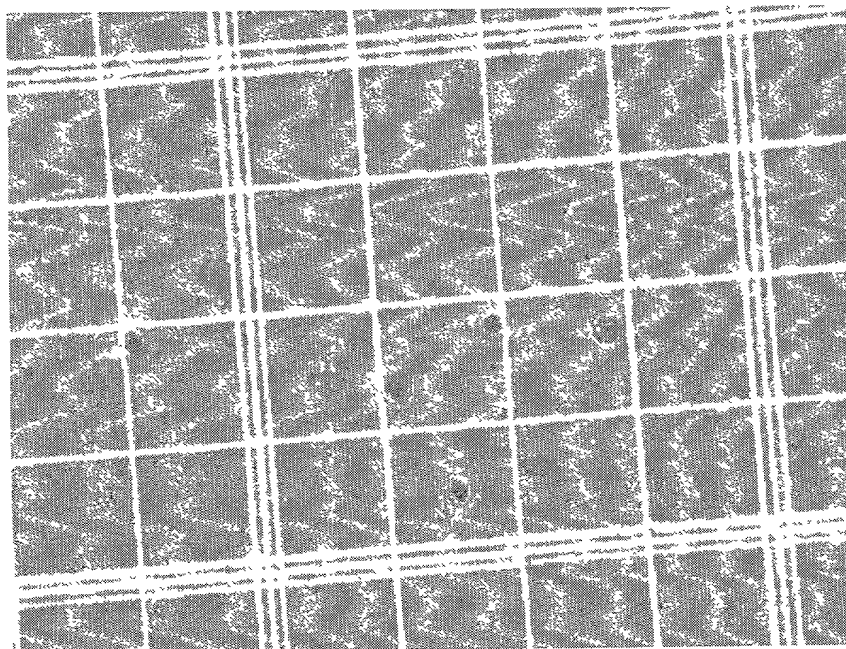
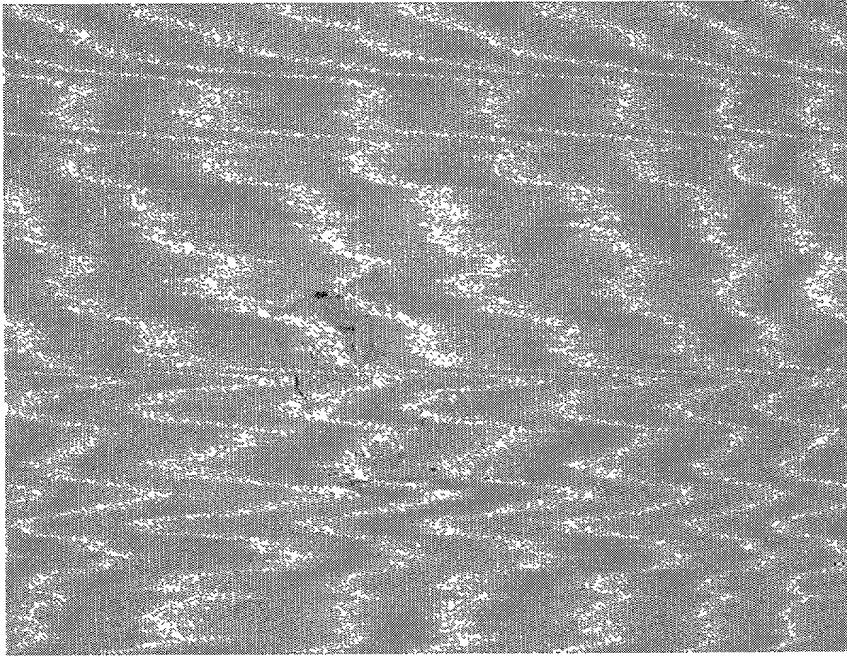
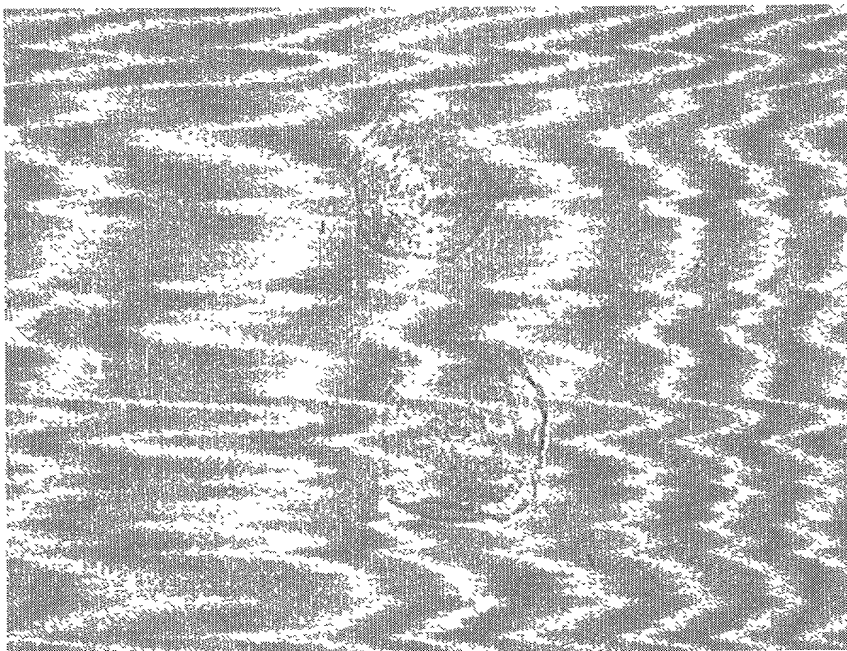


FIG. 5B

**FIG. 6**



**FIG. 6A**



**FIG. 6B**

Docket No.  
P-4762

# Declaration and Power of Attorney For Patent Application

## English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

**A COMPOSITION FOR PROVIDING LONG TERM STABILITY TO CELLS FOR DIAGNOSTIC TESTING**

the specification of which

(check one)

☒ is attached hereto.

☐ was filed on \_\_\_\_\_ as United States Application No. or PCT International

Application Number \_\_\_\_\_

and was amended on \_\_\_\_\_

(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)

Priority Not Claimed

_____ (Number)	_____ (Country)	_____ (Day/Month/Year Filed)	<input type="checkbox"/>
_____ (Number)	_____ (Country)	_____ (Day/Month/Year Filed)	<input type="checkbox"/>
_____ (Number)	_____ (Country)	_____ (Day/Month/Year Filed)	<input type="checkbox"/>



I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

\_\_\_\_\_  
(Application Serial No.)

\_\_\_\_\_  
(Filing Date)

\_\_\_\_\_  
(Application Serial No.)

\_\_\_\_\_  
(Filing Date)

\_\_\_\_\_  
(Application Serial No.)

\_\_\_\_\_  
(Filing Date)

I hereby claim the benefit under 35 U. S. C. Section 120 of any United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, C. F. R., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

\_\_\_\_\_  
(Application Serial No.)

\_\_\_\_\_  
(Filing Date)

\_\_\_\_\_  
(Status)  
(patented, pending, abandoned)

\_\_\_\_\_  
(Application Serial No.)

\_\_\_\_\_  
(Filing Date)

\_\_\_\_\_  
(Status)  
(patented, pending, abandoned)

\_\_\_\_\_  
(Application Serial No.)

\_\_\_\_\_  
(Filing Date)

\_\_\_\_\_  
(Status)  
(patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. *(list name and registration number)*

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	Citizenship	
	Post Office Address	

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Sixth inventor's signature	Date
Residence	
Citizenship	
Post Office Address	